



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

# The impact of 27-hydroxycholesterol on endometrial cancer proliferation

### Citation for published version:

Gibson, DA, Collins, F, Cousins, FL, Esnal-Zufiaurre, A & Saunders, PT 2018, 'The impact of 27-hydroxycholesterol on endometrial cancer proliferation', *Endocrine-Related Cancer*.  
<https://doi.org/10.1530/ERC-17-0449>

### Digital Object Identifier (DOI):

[10.1530/ERC-17-0449](https://doi.org/10.1530/ERC-17-0449)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Endocrine-Related Cancer

### General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



**The impact of 27-hydroxycholesterol on endometrial cancer proliferation**

*Running title – 27-hydroxycholesterol in Endometrial Cancer*

Authors: Douglas A Gibson<sup>1</sup>, Frances Collins<sup>1</sup>, Fiona L Cousins<sup>1\*</sup>, Arantza Esnal Zufiaurre<sup>1</sup>  
and Philippa T.K. Saunders<sup>1</sup>

Medical Research Council Centres for Inflammation Research<sup>1</sup>, The University of Edinburgh,  
Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh, EH16 4TJ. UK

\* Current address: The Ritchie Centre, Hudson Institute of Medical Research, 27-31 Wright  
Street, Clayton, Victoria, 3168, Australia.

**Word count: 4360**

**Key words: LXR, ER, 27-hydroxycholesterol, proliferation, endometrial cancer**

Corresponding Author:  
Dr Douglas Gibson,  
MRC Centre for Inflammation Research, The University of Edinburgh, Queen’s Medical  
Research Institute, 47 Little France Crescent, Edinburgh, EH16 4TJ, U.K.  
Tel: (+44)01312429196  
E-mail: d.a.gibson@ed.ac.uk.

24 **Abstract**

25 Endometrial cancer (EC) is the most common gynaecological malignancy. Obesity is a major  
26 risk factor for EC and is associated with elevated cholesterol. 27-Hydroxycholesterol (27HC)  
27 is a cholesterol metabolite that functions as an endogenous agonist for Liver X Receptor  
28 (LXR) and a selective estrogen receptor modulator (SERM). Exposure to estrogenic ligands  
29 increases risk of developing EC however the impact of 27HC on EC is unknown.

30 Samples of stage 1 EC (n=126) were collected from post-menopausal women undergoing  
31 hysterectomy. Expression of LXRs (*NR1H3*, LXR $\alpha$ ; *NR1H2*, LXR $\beta$ ) and enzymes required  
32 for the synthesis (*CYP27A1*) or breakdown (*CYP7B1*) of 27HC were detected in all grades of  
33 EC. Cell lines originating from well-, moderate- and poorly-differentiated endometrial cancers  
34 (Ishikawa, RL95, MFE 280 respectively) were used to assess the impact of 27HC or the LXR  
35 agonist GW3965 on proliferation or expression of a luciferase reporter gene under the  
36 control of LXR- or ER-dependent promoters (LXRE, ERE). Incubation with 27HC or GW3965  
37 increased transcription via LXRE in Ishikawa, RL95 and MFE 280 cells ( $p<0.01$ ). 27HC  
38 selectively activated ER-dependent transcription ( $p<0.001$ ) in Ishikawa cells and promoted  
39 proliferation of both Ishikawa and RL95 cells ( $p<0.001$ ). In MFE 280 cells 27HC did not alter  
40 proliferation but selective targeting of LXR with GW3965 significantly reduced cell  
41 proliferation ( $p<0.0001$ ).

42 These novel results suggest that 27HC can contribute to risk of EC by promoting  
43 proliferation of endometrial cancer epithelial cells and highlight LXR as a potential  
44 therapeutic target in the treatment of advanced disease.

**Introduction**

Endometrial cancer (EC) is the most common gynaecological malignancy and the fourth most common cancer in women in developed countries with incidence increasing in line with rising rates of obesity (reviewed in (Onstad, et al. 2016)). Obesity is a major modifiable risk factor for EC and is thought to contribute to increased risk of malignancy in part due to increased exposure to estrogens which enhance risk of aberrant proliferation within the endometrium (Sanderson, et al. 2017). Obesity is also associated with an adverse metabolic profile which is postulated to independently increase risk of EC (Trabert, et al. 2015).

A recent meta-analysis supported a positive association between dietary cholesterol consumption and endometrial cancer risk (Gong, et al. 2016). Notably, obesity also puts individuals at risk of developing an adverse, raised, cholesterol profile. Cholesterol metabolites such as the oxysterol 27-hydroxycholesterol (27HC) have been demonstrated to promote cancer growth and metastasis in studies on breast cancer (Nelson, et al. 2013; Wu, et al. 2013a); providing a plausible mechanistic link between increased adiposity and EC risk. 27HC is a primary metabolite of cholesterol, synthesised by the action of sterol 27-hydroxylase (CYP27A1) and metabolised by 25-hydroxycholesterol 7- $\alpha$ -hydroxylase (CYP7B1; [1]). 27HC acts as an endogenous agonist for the Liver X receptor (LXR), a ligand activated transcription factor involved in the regulation of cholesterol homeostasis. Two isoforms of LXR have been identified; LXR $\alpha$  (encoded by *NR1H3*) which is predominantly expressed in the liver, kidney and small intestine but exhibits low expression in other tissues, and LXR $\beta$  (encoded by *NR1H2*) which is ubiquitously expressed. Based largely on studies in breast cancer, LXRs have been proposed as a novel anti-cancer target and the LXR-selective agonists GW3965 and T0901317 are reported to decrease proliferation of LXR-expressing breast cancer cell lines (MCF7, T47D, MDA-MB231) as well as the prostate cancer cell line LNCaP (Kim, et al. 2010; Vedin, et al. 2009). To the best of our knowledge

71 LXR expression has not been reported in human EC tissues and the impact of either 27HC  
 72 or LXR agonists on the endometrium or endometrial malignancies is not known.

73 In addition to activating LXRs, 27HC can also bind estrogen receptors (ER)  
 74 (Umetani, et al. 2007) and acts as an endogenous selective estrogen receptor modulator  
 75 (SERM) (DuSell, et al. 2008). 27HC has diverse impacts and its SERM activity is reported to  
 76 be both tissue-selective and context-dependent. For example, 27HC acts as a competitive  
 77 antagonist of ERs expressed in the vasculature and can antagonise E2-mediated endothelial  
 78 cell migration and re-endothelialisation (Umetani et al. 2007). In contrast, in the absence of  
 79 E2, 27HC is reported to act as an agonist to ER $\alpha$  (ESR1) to increase cell adhesion and  
 80 expression of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFA) and  
 81 interleukin 6 (IL6) (Umetani, et al. 2014) by endothelial cells and macrophages. Notably,  
 82 27HC is also reported to increase proliferation of ER $\alpha$ -positive breast cancer cell lines and  
 83 promotes MCF7 tumour xenograft growth in mice by stimulating ER-dependent cell  
 84 proliferation (Wu et al. 2013a). Given selective LXR agonists have anti-proliferative effects  
 85 (Vedin et al. 2009), these studies suggest that proliferative effects of 27HC may be mediated  
 86 via ER and that relative expression of LXR or ER isoforms may define the impact of the  
 87 ligand.

88 ER isoforms are expressed in EC tissues and ER expression changes with disease  
 89 progression (Collins, et al. 2009). We have previously reported that ER $\alpha$  is readily detectable  
 90 in both epithelial and stromal cells in well-differentiated cancers but is significantly reduced in  
 91 poorly-differentiated cancers. In contrast, expression of ESR2 variants (ER $\beta$ 1, 2, 5) was  
 92 readily detected in well, moderate and poorly differentiated stage 1 ECs (Collins et al. 2009).  
 93 We therefore postulated that 27HC might have distinct effects in EC depending on the  
 94 bioavailability of ER isoforms present at different stages of disease progression.

95 Obesity and the metabolic syndrome are both associated with an increased risk of  
 96 developing pre-malignant and malignant endometrial disease (Sanderson et al. 2017) but  
 97 whether the cholesterol metabolite 27HC has an impact on endometrial cancer  
 98 risk/progression is not known. In the current study we assessed the expression of the

99 enzymes required for synthesis (CYP27A1) and breakdown (CYP7B1) of 27HC and  
100 assessed expression of the cognate receptors LXR $\alpha$  and LXR $\beta$  in primary human stage I  
101 endometrial adenocarcinomas (n=126) and postmenopausal endometrial controls (n=9). The  
102 impact of 27HC and the LXR-selective agonist GW3965 on ERE- and LXRE-dependent  
103 expression of a reporter gene, as well as cellular proliferation, was assessed in three  
104 endometrial cancer cell lines which phenocopy well-, moderately- and poorly-differentiated  
105 stage I endometrial cancers. Our novel findings demonstrate that 27HC can alter responses  
106 in endometrial cancer cells and highlight LXR as a potential therapeutic target. Taken  
107 together our findings suggest increased exposure to 27HC may increase risk of development  
108 and progression of endometrial cancer.

## **Materials and methods**

### **Human tissue samples**

Endometrial adenocarcinoma tissue was collected from post-menopausal women undergoing total abdominal hysterectomy who had been previously diagnosed to have endometrioid adenocarcinoma of the endometrium; they had received no treatment before surgery (supplementary table 1). Written informed consent was obtained from all subjects prior to surgery, and ethical approval was granted by the Lothian Research Ethics Committee (LREC1999/6/4). Methods were carried out in accordance with NHS Lothian Tissue Governance guidelines. All endometrial cancers were confined to the uterus (International Federation of Obstetrics and Gynaecology, FIGO, stage 1 as described in (Collins et al. 2009)). Diagnosis of adenocarcinoma was confirmed histologically by an experienced gynaecological pathologist and tissues were further graded as well differentiated (G1), moderately differentiated (G2) or poorly differentiated (G3). Samples were anonymised and patient follow-up information was not available. However, survival statistics for stage 1 EC in the UK are reported as 99% 1-year survival and 95.3% 5-year survival (Cancer Research UK; <http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/uterine-cancer/> accessed November 2017) and Information Services Division Scotland figures, which cover the stage 1 EC samples collected in the current study, report 92.9% 1-year survival and 83.2% 5-year survival for all uterine cancers (<http://www.isdscotland.org/Health-Topics/Cancer/Cancer-Statistics/Female-Genital-Organ/#uterus> accessed November 2017).

Postmenopausal controls (n=9) were obtained from women undergoing surgery for non-malignant gynaecological conditions. None of the women were receiving hormonal therapy. A total of 126 EC tissue samples were analysed; 3 samples per grade were assessed for immunohistochemistry and n=30 well differentiated cancers, n=64 moderately differentiated and n=32 poorly differentiated samples were assessed for qPCR studies. A minimum of 10

135 samples at each grade were analysed for each gene, detailed sample numbers are included  
136 in supplementary table 2. Tissue for immunohistochemistry was collected in neutral buffered  
137 formalin (NBF), RNA extraction samples were collected in RNALater (Qiagen, UK).

#### 138 Measurement of mRNA

139 Isolation of mRNAs, preparation of cDNAs, and analysis by qPCR was performed according  
140 to standard protocols (Bombail, et al. 2010); samples were quantified by relative standard  
141 curve method or by the comparative  $\Delta\Delta C_t$  method with *CYC* as internal control.  
142 Primers/probes are given in supplementary table 3.

#### 143 Immunohistochemistry

144 Single antibody immunohistochemistry using 3,3'-diaminobenzidine tetra-hydrochloride  
145 (DAB) detection was performed as described previously (Collins et al. 2009). Double  
146 immunofluorescence was carried out with antibodies directed against LXR or ER $\alpha$  and the  
147 proliferation marker Ki67. Details of antibodies and dilutions are provided in supplementary  
148 table 4. Primary antibodies were incubated at 4°C overnight. Antigen detection was  
149 performed using Tyramide signal amplification (Perkin Elmer) system followed by nuclear  
150 counterstaining with DAPI (4', 6-Diamidino-2-phenyl-indole dihydrochloride). Negative  
151 controls were incubated in the absence of primary antibody but otherwise processed as  
152 above; no staining was detected in no primary controls for any of the antibodies used (not  
153 shown). Images were captured using a LSM 710 Confocal microscope (Zeiss) at x40  
154 magnification.

#### 155 Cell Cultures

156 Three endometrial adenocarcinoma cell lines representative of well-, moderately- or poorly-  
157 differentiated cancers were used. Ishikawa cells were obtained from the European Collection  
158 of Cell Culture (ECACC no 99040201, Wiltshire, UK). This cell line was originally derived  
159 from a well-differentiated adenocarcinoma of a 39 year-old woman (Nishida, et al. 1985) and



reported to express both ER $\alpha$  and ER $\beta$  protein (Johnson, et al. 2007). RL95-2 cells (ATCC® CRL-1671™; hereafter RL95) were originally derived from a Grade 2 moderately differentiated endometrial adenocarcinoma (Way, et al. 1983) and reported to express both ER $\alpha$  and ER $\beta$  protein (Li, et al. 2014; Yang, et al. 2008). MFE-280 (ECACC no 98050131) were derived from a recurrent, poorly differentiated, endometrial adenocarcinoma and have low/undetectable expression of ER $\alpha$  and ER $\beta$ . Cells were maintained in DMEM/F12 (Sigma) supplemented with 10% FBS, 100U penicillin, streptomycin and 0.25  $\mu$ g/ml fungizone (Invitrogen, Paisley, UK) at 37°C in 5% CO<sub>2</sub>. Media for RL95 was supplemented with 0.005mg/ml Insulin (Sigma). Cells were incubated with 27-hydroxycholesterol (27HC; Tocris Cat. No. 3907) using stocks diluted in ethanol to give final concentrations ranging from 10<sup>-5</sup>M to 10<sup>-8</sup>M or GW 3965 hydrochloride (GW; Tocris Cat. No. 2474) using stocks diluted in DMSO to give final concentrations ranging from 10<sup>-5</sup>M to 10<sup>-8</sup>M. Some cultures were co-incubated with the anti-estrogen fulvestrant (ICI 182,780; Tocris Cat. No. 1047) diluted in DMSO at a final concentration of 10<sup>-6</sup>M. Appropriate vehicle control incubations were included in all studies. All cell lines were authenticated using the Promega PowerPlex 21 system (Eurofins Genomics, Ebersberg, Germany).

#### Reporter assays

An adenoviral vector containing a 3xERE-tk-luciferase reporter gene was prepared as described previously (Collins et al. 2009). Cells were cultured in DMEM without phenol red and containing charcoal stripped foetal calf serum (CSFCS) for 24 hours before being infected with Ad-ERE-Luc at a MOI of 25. Activation of LXR-dependent signal transduction was assessed according to manufacturer's instructions using reagents from the Cignal LXR Reporter Kit which includes positive and negative controls as well as a luciferase reporter gene under the control of tandem repeats of the LXR transcriptional response element (LXRE) (Qiagen, CCS-0041L).

Cells were treated for 24 hours and luciferase activities were determined using 'Bright Glo' reagents (Promega). Luminescence was measured using Fluostar Microplate Reader (BMG labtech) and fold change in luciferase activity calculated relative to vehicle control for each treatment.

#### Proliferation assays

The impact of treatments on cell proliferation was assessed using CyQUANT® Direct Cell Proliferation Assay (Thermo Fisher, C35011) according to manufacturer's instructions and nuclear fluorescence measured using Novostar Microplate Reader (BMG labtech). For each cell line investigated, cell number was quantified using a standard curve of known cell numbers and fold change in cell number calculated relative to vehicle control for each treatment.

#### Statistical analysis

Statistical analysis was performed using Graphpad prism. One-way ANOVA was used to determine significance between treatments in data that were normally distributed. Non-parametric testing was utilised where sample sizes were insufficient to confirm normality of data distribution; Kruskal-Wallis test was used to assess differences between treatments. Where data were analysed as fold-change significance was tested using one sample t test and a theoretical mean of 1. Criterion for significance was  $p < 0.05$ . All data are presented as mean  $\pm$  SEM.

### **Results**

*Enzymes that regulate bioavailability of 27-hydroxycholesterol and its cognate receptor LXR are expressed in endometrial cancer.*

Messenger RNAs encoded by *CYP7B1* and *CYP27A1* were detected in all cancer grades (Figure 1A and B); expression of *CYP7B1* was significantly lower in poorly-differentiated cancers compared to moderately differentiated cancers ( $p < 0.05$ ). Relative expression of

210 *CYP27A1* tended to be higher in poorly differentiated cancers but this was not significant.  
 211 We next assessed relative expression of mRNAs encoding the LXR receptors known to bind  
 212 27HC: *NR1H3* (LXR $\alpha$ ) and *NR1H2* (LXR $\beta$ ) were detected in all cancer grades (Figure 1C  
 213 and D). Expression of *NR1H3* was significantly lower in moderately-differentiated cancers  
 214 compared to post-menopausal controls ( $p < 0.01$ ). Expression of *NR1H2* did not change  
 215 between sample groups.

216 *Immunolocalisation of LXR and the proliferation marker Ki67 in endometrial cancer tissue*  
 217 *sections.*

218 The expression of LXR in endometrial cancer tissue sections was assessed by  
 219 immunohistochemistry using an antibody that detected both isoforms of LXR (mouse anti-  
 220 LXR; sc-271064). LXR was readily detected in well-, moderately- or poorly-differentiated  
 221 cancers and was immunolocalised to both stromal and epithelial cells (supplementary figure  
 222 1). To assess if LXR expression was associated with cell proliferation within endometrial  
 223 cancer tissue we performed double immunofluorescence staining for both LXR and the  
 224 proliferation marker Ki67 (Figure 2). In well-differentiated cancers (Figure 2A), nuclear  
 225 immunoexpression of Ki67 (red staining) was detected which co-localised (yellow arrows)  
 226 with LXR expression (green staining, note that single channel views show that the intensity  
 227 of LXR staining varied between cells). Whilst careful evaluation of single channel views  
 228 confirmed that the majority of LXR-positive cells were also immunopositive for Ki67 some  
 229 cells were Ki67-negative (white arrows). In contrast, in moderately-differentiated cancers  
 230 (Figure 2B) both markers were detected but few cells appeared to co-localise (yellow  
 231 arrows) although LXR-positive cells (white arrows) were found in close association with  
 232 proliferating cells. In poorly differentiated cancers (Figure 2C), few cells expressed both  
 233 markers. Ki67-positive cells were clustered in regions with limited LXR expression and no  
 234 co-expression of LXR and Ki67 was detected. LXR<sup>+</sup>Ki67<sup>-</sup> cells (white arrows) were detected  
 235 close to Ki67<sup>+</sup> cells. We also assessed the expression of ER $\alpha$  and Ki67 in endometrial  
 236 cancer tissues (supplementary figure 2) as this receptor is implicated in regulation of

proliferation in normal endometrium (Frasor, et al. 2003; Lubahn, et al. 1993). Consistent with our previous study, ER $\alpha$  was not detected in the poorly differentiated cancers (Collins et al. 2009) and immunoexpression of Ki67 was clearly independent of ER $\alpha$  with an increase in abundance of positive nuclei in poor (sample codes 910/2178) as compared to well or moderately differentiated tissue where co-localisation of ER $\alpha$  and Ki67 was readily detected.

*27HC activates LXRE- and ERE-dependent transcription in endometrial epithelial cancer cells and alters proliferation of endometrial cancer cells*

Having demonstrated expression of enzymes and receptors required for 27HC signalling, we extended our observational study by exploring the impact of the ligand on endometrial epithelial cancer cell lines chosen to model well-, moderately- or poorly-differentiated stage I cancers; Ishikawa, RL95 and MFE 280. Protein expression of both LXR isoforms was confirmed by western blot in all cell lines studied (supplementary figure 3A and 3B). We assessed the mRNA expression of LXRs in these cell lines and found that their expression phenocopied that found in tissue samples (Supplementary figure 3). *NR1H3* mRNA expression was significantly decreased in RL95 (moderately-differentiated) cells compared to MFE 280 (poorly-differentiated;  $p < 0.01$ ; Supplementary figure 3C). Consistent with tissue mRNA expression patterns *NR1H2* was not different between cell lines (Supplementary figure 3D). Messenger RNAs encoded by both ER genes; ER $\alpha$  (*ESR1*) and ER $\beta$  (*ESR2*; ER $\beta$ 1 specific primers) were detected in all of the cell lines (Supplementary figure 4). *ESR1* mRNAs were significantly reduced in RL95 and MFE280 compared to Ishikawa cells (Supplementary figure 4A) consistent with patterns of expression in intact tissue (supplementary figure 2). *ESR2* mRNA was significantly reduced in MFE280 cells compared to Ishikawa (Supplementary figure 4B). As 27HC is both an endogenous agonist for LXR and a SERM, the impact of 27HC on LXRE- and ERE-dependent transcription was investigated in the endometrial cancer cell lines. 27HC significantly increased LXRE-dependent transcription in a dose-dependent manner in all 3 cell lines and was maximally stimulated by  $10^{-5}$ M 27HC (Figure 3 A-C). In contrast, 27HC only stimulated ERE-dependent transcription

in Ishikawa cells (Figure 3D) at  $10^{-8}$ M ( $p<0.01$ ) and  $10^{-7}$ M ( $p<0.0001$ ). The impact of 27HC was abrogated by co-incubation with the anti-estrogen Fulvestrant (ICI 182,780) consistent with ER dependence. In contrast to Ishikawa cells, 27HC had little impact on ERE-dependent transcription in RL95 (Figure 3E) and MFE280 cells (Figure 3F). As 27HC could activate both ERE- and LXRE-promoters, we assessed its impact on cell proliferation (Figure 3 G-I). 27HC induced proliferation of Ishikawa cells at concentrations ranging from  $10^{-8}$ M to  $10^{-6}$ M ( $p<0.01$ ) but this was inhibited at the highest concentration ( $10^{-5}$ M,  $p<0.0001$ ). 27HC significantly increased proliferation in RL95 cells at concentrations of  $10^{-7}$ M ( $p<0.001$ ) or greater. In contrast, 27HC did not alter proliferation of MFE 280 cells at any of the concentrations investigated. Neither RL95 nor MFE280 cell lines expressed *CYP7B1* (supplementary figure 3E and 3F) precluding the potential for in vitro metabolism limiting cell responses to 27HC in these cell lines.

*Targeting LXR with the synthetic agonist GW3965 activates LXRE-dependent transcription and alters cell proliferation in a cell specific manner.*

Incubation of cells with the LXR-selective agonist GW3965 significantly increased LXRE-dependent transcription in a dose-dependent manner (Figure 4) consistent with expression of LXRs in the endometrial cancer cell lines (Supplementary Figure 3). In contrast to 27HC, GW3965 significantly and robustly increased LXRE-dependent transcription at concentrations  $\geq 10^{-8}$ M in Ishikawa (Figure 4A) and RL95 (Figure 4B) and  $\geq 10^{-7}$ M in MFE280 cells (Figure 4C). Although LXR reporter responses were similar in the different cell lines, proliferation responses were strikingly different. In Ishikawa cells, treatment with GW3965 at concentrations  $10^{-8}$ M ( $p<0.01$ ) and  $10^{-5}$ M ( $p<0.01$ ) significantly increased proliferation (Figure 4D). In contrast, GW3965 significantly and robustly decreased cell proliferation at all concentrations investigated in both RL95 (Figure 4E) and MFE 280 cells (Figure 4F).

**Discussion**

To date, no study has assessed the association between the cholesterol metabolite 27HC and EC. EC incidence rates have increased by ~50% since the early 1990s and approximately 57% of endometrial cancers in the United States have been attributed to being overweight or obese (Cancer Research UK; <http://www.cancerresearchuk.org> - accessed November 2017, and (Calle and Kaaks 2004)). Although increased exposure to adipose-derived estrogens is believed to increase aberrant proliferation within the endometrium (Zhao, et al. 2016), recent evidence supports an independent role for obesity-associated metabolic factors in modulating EC risk. Notably, both elevated triglycerides and increased dietary cholesterol consumption are reported to be associated with increased EC risk (Gong et al. 2016; Lindemann, et al. 2009). Importantly, concentrations of the cholesterol metabolite 27HC are increased in postmenopausal women (Burkard, et al. 2007) and are associated with increased risk of breast cancer. Several studies have identified that 27HC has an adverse impact on breast cancer (Nelson et al. 2013; Wu et al. 2013a) but whether 27HC can affect EC has not previously been investigated.

In light of these studies we hypothesised that 27HC signalling could contribute to the aetiology of endometrial cancer and influence disease progression and we investigated this using both archival human tissue as well as cell lines that are derived from different grades of EC. We obtained new evidence for expression of the enzymes required for the both the synthesis (CYP27A1) and breakdown (CYP7B1) of 27HC. As concentrations of *CYP7B1* mRNAs were significantly decreased in poorly- compared to moderately-differentiated cancers and expression of *CYP27A1* did not change significantly across EC grades; we believe this would favour increased bioavailability of 27HC with increasing grade. These findings appear to parallel those reported for ER+ breast cancer where decreased expression of CYP7B1 and increased CYP27A1 has been reported in tumours compared to normal breast tissues (Wu et al. 2013a). Furthermore, we found that the endogenous

receptor for 27HC, LXR, was immunolocalised to stage 1 cancers and was expressed throughout the tissue and localised to the nuclei of both stromal and epithelial cells.

We sought to establish if 27HC could alter responses in endometrial cancer cells by acting via its cognate receptor, LXR, or via estrogen receptors which are known to regulate endometrial proliferation. 27HC activated LXR-dependent transcription in all cell lines tested. In contrast, we found that 27HC activated ERE-dependent reporter gene expression in well-differentiated cancer cells (Ishikawa; ER $\alpha$ +ER $\beta$ +) but not in those from moderately- (RL95; ER $\alpha^{\text{low}}$ ER $\beta$ +) or poorly-differentiated cancers (MFE280; ER $\alpha^{\text{low}}$ ER $\beta^{\text{low}}$ ). However, 27HC increased proliferation of both Ishikawa and RL95 cells but not MFE280 cells consistent with reported ER expression in these cell lines (Johnson et al. 2007; Li et al. 2014; Yang et al. 2008). Our immunohistochemistry analysis (supplementary figure 2) supported these in vitro findings. We found that the proliferation marker Ki67 co-localised with ER $\alpha$  in well- and moderately-differentiated cancers consistent with a key role for this receptor in mediating endometrial epithelial cell proliferation (Frasor et al. 2003; Lubahn et al. 1993). In poorly-differentiated cancers, ER $\alpha$  was not detected consistent with previous reports (Collins et al. 2009). It has been reported that 27HC, acting as a SERM, can impact on ER $\alpha$ - or ER $\beta$ 1-dependent regulation of cell function (He and Nelson 2017) and the estrogenic effects of 27HC could therefore be mediated via either ER isoform in EC cells. In endometrial endothelial cells, which express ER $\beta$  but not ER $\alpha$ , estrogenic effects are mediated via ER $\beta$  tethered to Sp1 and not via direct binding to ERE (Greaves, et al. 2013). Furthermore, it has been reported that 27HC promotes proliferation of ER $\alpha$ -positive LNCaP prostate cancer cells via ER $\beta$  (Lau, et al. 2000) (Raza, et al. 2017) which may account for the apparent discrepancy between ERE reporter assay and cell proliferation responses in RL95 cells observed in the current study. Taken together, these findings reveal the potential for 27HC generated within the EC tissue microenvironment to influence ER-dependent transcription and proliferation via ERs expressed in early grade stage 1 EC.

340 Although the association between ERs and endometrial proliferation is well recognised,  
341 there is limited data investigating the role of LXR in this process. Expression of LXR $\alpha$  and  
342 LXR $\beta$  mRNA has been previously reported in endometrium and myometrium of mice  
343 (Mouzat, et al. 2007) and 27HC is reported to increase mouse uterine weight, consistent with  
344 an uterotrophic action, however, whether this was mediated via ER or LXR was not  
345 investigated (Wu, et al. 2013b). In mice, targeted ablation of the receptor subtypes revealed  
346 that *Lxr $\alpha$* <sup>-/-</sup> but not *Lxr $\beta$* <sup>-/-</sup> females had reduced endometrial areas compared to wildtype  
347 mice consistent with a role for LXR $\alpha$  in promoting endometrial growth/proliferation in that  
348 species (Mouzat et al. 2007). In the current study we found that LXR co-localised with the  
349 proliferation marker Ki67 in well-differentiated but not moderate- or poorly differentiated EC  
350 tissues. In vitro assays verified this finding as the synthetic LXR agonist GW3965 had a cell-  
351 selective impact on the EC cell lines. In Ishikawa cells GW3965 increased proliferation,  
352 whereas in RL95 and MFE280 cells equimolar concentrations of agonist blocked  
353 proliferation. Given that LXR expression was detected in all grades of endometrial cancer  
354 this may suggest LXR could be an effective therapeutic target in some endometrial cancers,  
355 albeit in a grade-dependent context. Indeed, GW3965, is reported to abrogate E2-mediated  
356 increases in MCF7 breast cancer cell proliferation and has been proposed as an anti-  
357 proliferative ligand in this context (Vedin et al. 2009).

358 LXR classically acts as a heterodimeric partner of retinoid X receptor (RXR). RXR is  
359 expressed in the nuclei of endometrial epithelial cells throughout the menstrual cycle  
360 (Fukunaka, et al. 2001) as well as in endometrial cancer tissues (Nickkho-Amiry, et al.  
361 2012). Interestingly, LXR-RXR functions as a 'permissive' heterodimer and binding of either  
362 an LXR agonist or the RXR agonist 9-*cis* retinoic acid activates transcription, while agonism  
363 of both dimer partners has an additive effect on activation. Assessment of RXR isoforms in  
364 the cell lines used in the current study demonstrated differential expression of RXRs in  
365 Ishikawa, RL95 and MFE280 cells which may account for the distinct responses of these cell  
366 lines in response to GW3965 treatment (supplementary figure 5). *NR2B1* (RXR $\alpha$ ) mRNA



expression was greatest in RL95 cells while *NR2B2* (RXR $\beta$ ) was detected in all cell lines. Notably, mRNA expression of *NR2B3* (RXR $\gamma$ ) was not detected in RL95 cells but was abundant in MFE280 cells. Whether changes in the constitution of the receptor isoforms that contribute to the LXR:RXR heterodimer affect responses requires further investigation, however, previous studies demonstrate that targeting retinoid signalling may affect proliferation of EC cells. Notably, retinoic acid (RA) signalling via retinoic acid receptor (RAR) and RXR is reported to inhibit Ishikawa cell proliferation by inducing cell cycle arrest (Cheng, et al. 2011) and fenretinide, a synthetic derivative of RA, induced apoptosis of Ishikawa cells (Mittal, et al. 2014). These results suggest targeting LXR-dependent signalling with LXR and/or RXR agonists could inhibit proliferation in EC and cancer progression.

Changes in the local inflammatory environment that occur during development and progression of endometrial cancer may also increase exposure to 27HC due to infiltration of inflammatory cells. We have previously demonstrated that infiltration of immune cells is increased in endometrial cancer tissues compared to controls. Notably, the numbers of macrophages, neutrophils and dendritic cells were significantly increased in EC tissues (Wallace, et al. 2010) consistent with 27HC-dependent increases in migration of bone marrow-derived CD11b<sup>+</sup> cells reported in in vitro assays (Raccosta, et al. 2013). In addition, 27HC increases secretion of CCL2 from macrophages which enhances recruitment of monocytes (Kim, et al. 2013) and can also upregulate ER-dependent expression of pro-inflammatory genes (Umetani et al. 2014). Notably, as Cyp27a1 is reported to be abundant in macrophages [2], these cells may also contribute to an increase in 27HC within the tumour microenvironment. In support of this idea, increased 27HC concentrations have been reported in breast cancer tumours (Wu et al. 2013b) and increased concentrations of cholesterol have been reported in tumours of various cancer types although they have not been directly measured in EC. 27HC can also promote secretion of TNFA and IL6 from macrophages and TNFA is reported to increase proliferation of human endometrial glandular epithelial cells (Nair, et al. 2013). Thus, although in the current study we only investigated

the direct impact of 27HC on proliferation of endometrial cancer epithelial cells, 27HC may also exacerbate changes within the tissue microenvironment by modulating inflammatory responses and this merits further investigation in animal models.

Summary

In the current study we provide the first evidence to support a mechanistic link between exposure to elevated cholesterol, biosynthesis of 27HC and EC. Analysis of human stage 1 endometrial adenocarcinomas revealed expression of the key metabolising enzymes of 27HC was altered in EC consistent with increased exposure to 27HC as EC progresses from well- to poorly-differentiated. Although survival rates for EC are high, incidence rates are increasing in line with rates of obesity and a rising incidence in pre- and peri-menopausal women creates unique therapeutic challenges. Based on our novel findings, we propose that exposure to 27HC may influence disease development/progression by activating ER-dependent pathways to increase epithelial cell proliferation. These results suggest strategies that seek to limit exposure to 27HC through lifestyle modification, lipid-lowering drugs such as statins or novel therapeutics that target 27HC synthesis (CYP27A1 inhibitors) may be effective in reducing endometrial proliferation in women at increased risk of developing EC. Taken together our novel findings suggest that altered cholesterol metabolism, and aberrant exposure to 27HC, may contribute to the development and/or progression of endometrial cancer.

Acknowledgements

We thank Dr Alistair Williams for histological evaluations of endometrial tissue and Prof Richard Anderson and clinical support team for collection of tissues. We thank members of PTKS laboratory for technical support. Studies undertaken in the Saunders laboratory were supported by MRC Programme Grant G1100356/1 (PTKS) and CRUK development fund (DAG and PTKS). The authors have no conflicts of interest to disclose.

Author contributions

Gibson et al *27-hydroxycholesterol in Endometrial Cancer*

420 Experimental design; DAG, FC & PTKS, experimental procedures; DAG, FC, AEZ, FLC,  
421 manuscript preparation; DAG, FC & PTKS.

422 **References**

- 423 Bombail V, Gibson DA, Collins F, MacPherson S, Critchley HO & Saunders PT 2010 A Role for the  
 424 orphan nuclear receptor estrogen-related receptor alpha in endometrial stromal cell decidualization  
 425 and expression of genes implicated in energy metabolism. *J Clin Endocrinol Metab* **95** E224-228.
- 426 Burkard I, von Eckardstein A, Waeber G, Vollenweider P & Rentsch KM 2007 Lipoprotein distribution  
 427 and biological variation of 24S- and 27-hydroxycholesterol in healthy volunteers. *Atherosclerosis* **194**  
 428 71-78.
- 429 Calle EE & Kaaks R 2004 Overweight, obesity and cancer: epidemiological evidence and proposed  
 430 mechanisms. *Nat Rev Cancer* **4** 579-591.
- 431 Cheng YH, Utsunomiya H, Pavone ME, Yin P & Bulun SE 2011 Retinoic acid inhibits endometrial  
 432 cancer cell growth via multiple genomic mechanisms. *J Mol Endocrinol* **46** 139-153.
- 433 Collins F, MacPherson S, Brown P, Bombail V, Williams AR, Anderson RA, Jabbour HN & Saunders PT  
 434 2009 Expression of oestrogen receptors, ERalpha, ERbeta, and ERbeta variants, in endometrial  
 435 cancers and evidence that prostaglandin F may play a role in regulating expression of ERalpha. *BMC*  
 436 *Cancer* **9** 330.
- 437 DuSell CD, Umetani M, Shaul PW, Mangelsdorf DJ & McDonnell DP 2008 27-hydroxycholesterol is an  
 438 endogenous selective estrogen receptor modulator. *Mol Endocrinol* **22** 65-77.
- 439 Frasor J, Barnett DH, Danes JM, Hess R, Parlow AF & Katzenellenbogen BS 2003 Response-specific  
 440 and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the  
 441 uterus. *Endocrinology* **144** 3159-3166.
- 442 Fukunaka K, Saito T, Wataba K, Ashihara K, Ito E & Kudo R 2001 Changes in expression and  
 443 subcellular localization of nuclear retinoic acid receptors in human endometrial epithelium during  
 444 the menstrual cycle. *MHR: Basic science of reproductive medicine* **7** 437-446.
- 445 Gong TT, Li D, Wu QJ & Wang YZ 2016 Cholesterol consumption and risk of endometrial cancer: a  
 446 systematic review and dose-response meta-analysis of observational studies. *Oncotarget* **7** 16996-  
 447 17008.
- 448 Greaves E, Collins F, Critchley HO & Saunders PT 2013 ERbeta-dependent effects on uterine  
 449 endothelial cells are cell specific and mediated via Sp1. *Hum Reprod* **28** 2490-2501.
- 450 He S & Nelson ER 2017 27-Hydroxycholesterol, an endogenous selective estrogen receptor  
 451 modulator. *Maturitas* **104** 29-35.
- 452 Johnson SM, Maleki-Dizaji M, Styles JA & White IN 2007 Ishikawa cells exhibit differential gene  
 453 expression profiles in response to oestradiol or 4-hydroxytamoxifen. *Endocr Relat Cancer* **14** 337-  
 454 350.
- 455 Kim KH, Lee GY, Kim JI, Ham M, Won Lee J & Kim JB 2010 Inhibitory effect of LXR activation on cell  
 456 proliferation and cell cycle progression through lipogenic activity. *Journal of Lipid Research* **51** 3425-  
 457 3433.
- 458 Kim SM, Lee SA, Kim BY, Bae SS, Eo SK & Kim K 2013 27-Hydroxycholesterol induces recruitment of  
 459 monocytic cells by enhancing CCL2 production. *Biochem Biophys Res Commun* **442** 159-164.
- 460 Lau K-M, LaSpina M, Long J & Ho S-M 2000 Expression of Estrogen Receptor (ER)- $\alpha$  and ER- $\beta$  in  
 461 Normal and Malignant Prostatic Epithelial Cells: Regulation by Methylation and Involvement in  
 462 Growth Regulation. *Cancer Res* **60** 3175-3182.
- 463 Li S, Hu R, Wang C, Guo F, Li X & Wang S 2014 miR-22 inhibits proliferation and invasion in estrogen  
 464 receptor alpha-positive endometrial endometrioid carcinomas cells. *Mol Med Rep* **9** 2393-2399.
- 465 Lindemann K, Vatten LJ, Ellstrom-Eng M & Eskild A 2009 Serum lipids and endometrial cancer risk:  
 466 results from the HUNT-II study. *Int J Cancer* **124** 2938-2941.
- 467 Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS & Smithies O 1993 Alteration of reproductive  
 468 function but not prenatal sexual development after insertional disruption of the mouse estrogen  
 469 receptor gene. *Proc Natl Acad Sci U S A* **90** 11162-11166.
- 470 Mittal N, Malpani S, Dyson M, Ono M, Coon JS, Kim JJ, Schink JC, Bulun SE & Pavone ME 2014  
 471 Fenretinide: A Novel Treatment for Endometrial Cancer. *PLOS ONE* **9** e110410.

- Mouzat K, Prod'homme M, Volle DH, Sion B, Dechelotte P, Gauthier K, Vanacker JM & Lobaccaro JM 2007 Oxysterol nuclear receptor LXRbeta regulates cholesterol homeostasis and contractile function in mouse uterus. *J Biol Chem* **282** 4693-4701.
- Nair S, Nguyen H, Salama S & Al-Hendy A 2013 Obesity and the Endometrium: Adipocyte-Secreted Proinflammatory TNF alpha Cytokine Enhances the Proliferation of Human Endometrial Glandular Cells. *Obstet Gynecol Int* **2013** 368543.
- Nelson ER, Wardell SE, Jasper JS, Park S, Suchindran S & Howe MK 2013 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. *Science* **342**.
- Nickkho-Amiry M, McVey R & Holland C 2012 Peroxisome proliferator-activated receptors modulate proliferation and angiogenesis in human endometrial carcinoma. *Mol Cancer Res* **10** 441-453.
- Nishida M, Kasahara K, Kaneko M, Iwasaki H & Hayashi K 1985 [Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors]. *Nihon Sanka Fujinka Gakkai Zasshi* **37** 1103-1111.
- Onstad MA, Schmandt RE & Lu KH 2016 Addressing the Role of Obesity in Endometrial Cancer Risk, Prevention, and Treatment. *J Clin Oncol* **34** 4225-4230.
- Raccosta L, Fontana R, Maggioni D, Lanterna C, Villablanca EJ, Paniccia A, Musumeci A, Chiricozzi E, Trincavelli ML, Daniele S, et al. 2013 The oxysterol-CXCR2 axis plays a key role in the recruitment of tumor-promoting neutrophils. *The Journal of Experimental Medicine* **210** 1711-1728.
- Raza S, Meyer M, Goodyear C, Hammer KDP, Guo B & Ghribi O 2017 The cholesterol metabolite 27-hydroxycholesterol stimulates cell proliferation via ERβ in prostate cancer cells. *Cancer Cell International* **17** 52.
- Sanderson PA, Critchley HO, Williams AR, Arends MJ & Saunders PT 2017 New concepts for an old problem: the diagnosis of endometrial hyperplasia. *Hum Reprod Update* **23** 232-254.
- Trabert B, Wentzensen N, Felix AS, Yang HP, Sherman ME & Brinton LA 2015 Metabolic syndrome and risk of endometrial cancer in the United States: a study in the SEER-Medicare linked database. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **24** 261-267.
- Umetani M, Domoto H, Gormley AK, Yuhanna IS, Cummins CL & Javitt NB 2007 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. *Nat Med* **13**.
- Umetani M, Ghosh P, Ishikawa T, Umetani J, Ahmed M, Mineo C & Shaul PW 2014 The cholesterol metabolite 27-hydroxycholesterol promotes atherosclerosis via proinflammatory processes mediated by estrogen receptor alpha. *Cell Metab* **20** 172-182.
- Vedin L-L, Lewandowski SA, Parini P, Gustafsson J-Å & Steffensen KR 2009 The oxysterol receptor LXR inhibits proliferation of human breast cancer cells. *Carcinogenesis* **30** 575-579.
- Wallace AE, Gibson DA, Saunders PT & Jabbour HN 2010 Inflammatory events in endometrial adenocarcinoma. *J Endocrinol* **206** 141-157.
- Way DL, Grosso DS, Davis JR, Surwit EA & Christian CD 1983 Characterization of a new human endometrial carcinoma (RL95-2) established in tissue culture. *In Vitro* **19** 147-158.
- Wu Q, Ishikawa T, Sirianni R, Tang H, McDonald JG & Yuhanna IS 2013a 27-Hydroxycholesterol promotes cell-autonomous, ER-positive breast cancer growth. *Cell Rep* **5**.
- Wu Q, Ishikawa T, Sirianni R, Tang H, McDonald JG, Yuhanna IS, Thompson B, Girard L, Mineo C, Brekken RA, et al. 2013b 27-Hydroxycholesterol promotes cell-autonomous, ER-positive breast cancer growth. *Cell Rep* **5** 637-645.
- Yang JZ, O'Flatharta C, Harvey BJ & Thomas W 2008 Membrane ERα-dependent activation of PKCα in endometrial cancer cells by estradiol. *Steroids* **73** 1110-1122.
- Zhao H, Zhou L, Shangguan AJ & Bulun SE 2016 Aromatase expression and regulation in breast and endometrial cancer. *J Mol Endocrinol* **57** R19-R33.

**Figure legends**

**Figure 1. 27HC signalling pathway is expressed in endometrial cancer and altered**

**with disease severity.** The expression of *CYP7B1*, *CYP27A1*, *NR1H3* (LXR $\alpha$ ) and *NR1H2*

(LXR $\beta$ ) relative to internal control gene *CYC* was assessed by qPCR in postmenopausal

control endometrium (PM Ctrl) and in endometrial cancer tissue homogenates from well-,

moderately- and poorly-differentiated endometrial adenocarcinomas. Relative expression of

mRNAs encoding *CYP7B1* (**A**) were decreased in poorly differentiated cancers compared to

moderately differentiated cancers but *CYP27A1* was not significantly different (**B**). Relative

expression of mRNAs encoding *NR1H3* (**C**; LXR $\alpha$ ) were significantly decreased in

moderately-differentiated cancers compared to post-menopausal control tissues while

*NR1H2* (LXR $\beta$ ) was not significantly different (**D**). \* $p < 0.05$ , \*\* $p < 0.01$ . Kruskal-Wallis test with

multiple comparisons. PM,  $n = 9$ ; Well  $n = 12-30$ ; Mod,  $n = 42-64$ ; Poor,  $n = 23-32$ . All data are

presented as mean  $\pm$  SEM.

**Figure 2. Expression of LXR and the proliferation marker Ki67 in endometrial cancer.**

The expression of LXR (antibody identified both isoforms) and the proliferation marker Ki67

was assessed by immunohistochemistry in endometrial cancer tissue sections. In well-

differentiated cancers (**A**), LXR was expressed throughout the tissue and localised to the

nuclei of both stromal and epithelial cells (green staining). Nuclear immunoexpression of

Ki67 (red staining) was detected and co-localised with LXR expression (yellow arrows)

although some LXR-positive cells did not co-express Ki67 (white arrows). In moderately-

differentiated cancers (**B**) both markers were detected but did not appear to co-localise; only

few cells expressed both LXR and Ki67 (yellow arrows). Most LXR-positive cells did not co-

express Ki67 (white arrows). This was also true of poorly differentiated cancers (**C**), few cells

expressed both LXR and Ki67 (yellow arrows) although LXR-positive cells were found in

close association with proliferating cells (white arrows). Images representative of at least 3

different patients per cancer grade. Nuclear counterstain DAPI (grey). All scale bars 50 $\mu$ M.

**Figure 3. 27HC activates LXRE- and ERE-dependent transcription in endometrial epithelial cancer cells and alters proliferation.** The cholesterol metabolite 27-hydroxycholesterol (27HC) is the endogenous agonist for LXR and is also classified as selective estrogen receptor modulator. The impact of 27HC on LXRE- (**A-C**) and ERE-dependent (**D-F**) transcription was investigated by luciferase reporter assay in endometrial cancer cell lines; Ishikawa, RL95 and MFE280. 27HC significantly increased LXRE-dependent transcription in a dose-dependent manner in each endometrial cancer cell line. 27HC stimulated ERE-dependent transcription only at lower concentrations and was significantly increased by  $10^{-8}$ M 27HC ( $p<0.01$ ) and maximally stimulated by  $10^{-7}$ M 27HC ( $p<0.0001$ ). The 27HC effect was abrogated by co-incubation with the antiestrogen Fulvestrant (ICI 182,780; ICI) at all concentrations of 27HC (**D**). 27HC did not increase ERE-dependent transcription in RL95 (**E**) and was only increased by  $10^{-5}$ M 27HC ( $p<0.05$ ) in MFE280 cells (**F**). Cell proliferation was assessed by CyQuant® direct proliferation assay in each cell line (**G-I**). Proliferation of Ishikawa cells was increased by  $10^{-8}$ M ( $p<0.01$ ),  $10^{-7}$ M ( $p<0.01$ ) and  $10^{-6}$ M ( $p<0.01$ ) 27HC but decreased by  $10^{-5}$ M 27HC ( $p<0.0001$ ; **G**). Proliferation of RL95 cells was increased by  $10^{-7}$ M ( $p<0.001$ ),  $10^{-6}$ M ( $p<0.01$ ) and  $10^{-5}$ M ( $p<0.001$ ) 27HC (**H**). 27HC did not affect proliferation in MFE280 cells (**I**). \* $p<0.05$ , \*\* $p<0.01$ , \*\*\*  $p<0.001$ , \*\*\*\*  $p<0.0001$ . One sample t test and a theoretical mean of 1. All data are presented as mean  $\pm$  SEM.

**Figure 4. LXR agonist GW3965 activates LXRE-dependent transcription and alters proliferation in endometrial epithelial cancer cells.** The impact of the LXR synthetic agonist GW3965 on LXRE-dependent transcription (**A-C**) and on cell proliferation (**D-F**) was assessed in endometrial cancer cell lines; Ishikawa, RL95 and MFE280. GW3965 significantly increased LXRE-dependent transcription in a dose-dependent manner in each endometrial cancer cell line. GW3965 significantly increased LXRE-dependent transcription at all concentrations assessed and was maximally increased by  $10^{-6}$ M GW3965 in Ishikawa cells ( $p<0.0001$ ) (**A**) and RL95 cells ( $p<0.01$ ) (**B**). LXRE-dependent transcription and was not

54 increased by  $10^{-8}$ M GW3965 but maximally increased by  $10^{-5}$ M 27HC ( $p<0.001$ ) in MFE280  
55 cells (**C**). Cell proliferation was assessed by CyQuant® direct proliferation assay in each cell  
56 line (**D-F**). Proliferation of Ishikawa cells was increased by  $10^{-8}$ M ( $p<0.01$ ) and by  $10^{-5}$ M  
57 27HC ( $p<0.01$ ) (**D**). In contrast, proliferation of RL95 cells was decreased by  $10^{-8}$ M  
58 ( $p<0.001$ ),  $10^{-7}$ M ( $p<0.001$ ),  $10^{-6}$ M ( $p<0.01$ ) and  $10^{-5}$ M ( $p<0.05$ ) GW3965 (**E**). Proliferation of  
59 MFE280 cells was decreased by  $10^{-8}$ M ( $p<0.001$ ),  $10^{-7}$ M ( $p<0.0001$ ),  $10^{-6}$ M ( $p<0.0001$ ) and  
60  $10^{-5}$ M ( $p<0.05$ ) GW3965 (**I**). \* $p<0.05$ , \*\* $p<0.01$ , \*\*\*  $p<0.001$ , \*\*\*\*  $p<0.0001$ . One sample t  
61 test and a theoretical mean of 1. All data are presented as mean  $\pm$  SEM.

62



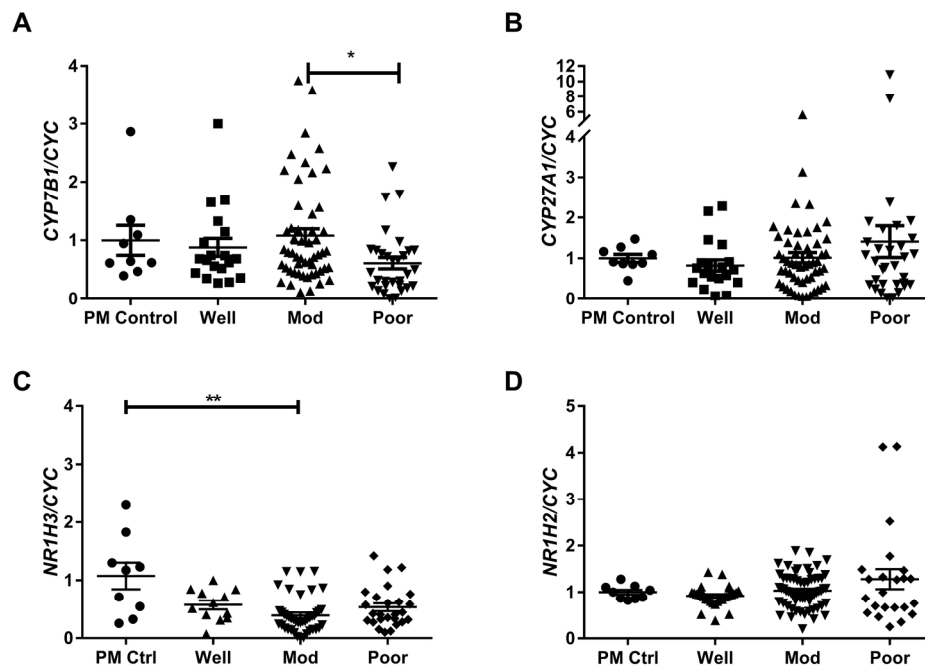


Figure 1. 27HC signalling pathway is expressed in endometrial cancer and altered with disease severity. The expression of CYP7B1, CYP27A1, NR1H3 (LXR $\alpha$ ) and NR1H2 (LXR $\beta$ ) relative to internal control gene CYC was assessed by qPCR in postmenopausal control endometrium (PM Ctrl) and in endometrial cancer tissue homogenates from well-, moderately- and poorly-differentiated endometrial adenocarcinomas. Relative expression of mRNAs encoding CYP7B1 (A) were decreased in poorly differentiated cancers compared to moderately differentiated cancers but CYP27A1 was not significantly different (B). Relative expression of mRNAs encoding NR1H3 (C; LXR $\alpha$ ) were significantly decreased in moderately-differentiated cancers compared to post-menopausal control tissues while NR1H2 (LXR $\beta$ ) was not significantly different (D). \* $p < 0.05$ , \*\* $p < 0.01$ . Kruskal-Wallis test with multiple comparisons. PM,  $n = 9$ ; Well  $n = 12-30$ ; Mod,  $n = 42-64$ ; Poor,  $n = 23-32$ . All data are presented as mean  $\pm$  SEM.

186x131mm (300 x 300 DPI)

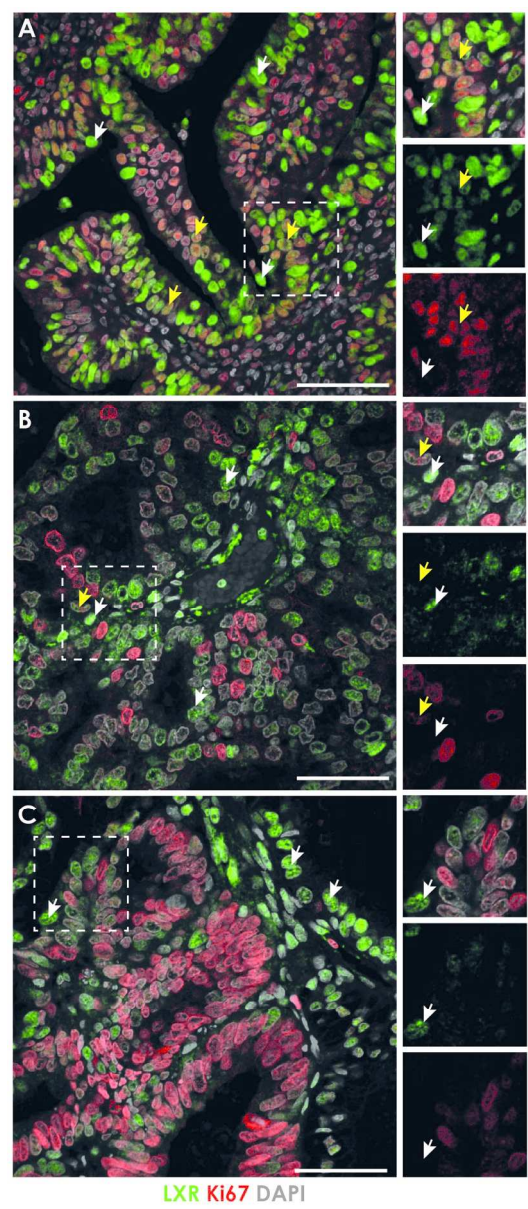


Figure 2. Expression of LXR and the proliferation marker Ki67 in endometrial cancer. The expression of LXR (antibody identified both isoforms) and the proliferation marker Ki67 was assessed by immunohistochemistry in endometrial cancer tissue sections. In well-differentiated cancers (A), LXR was expressed throughout the tissue and localised to the nuclei of both stromal and epithelial cells (green staining). Nuclear immunoexpression of Ki67 (red staining) was detected and co-localised with LXR expression (yellow arrows) although some LXR-positive cells did not co-express Ki67 (white arrows). In moderately-differentiated cancers (B) both markers were detected but did not appear to co-localise; only few cells expressed both LXR and Ki67 (yellow arrows). Most LXR-positive cells did not co-express Ki67 (white arrows). This was also true of poorly differentiated cancers (C), few cells expressed both LXR and Ki67 (yellow arrows) although LXR-positive cells were found in close association with proliferating cells (white arrows). Images representative of at least 3 different patients per cancer grade. Nuclear counterstain DAPI (grey). All scale bars 50µM.

101x230mm (300 x 300 DPI)

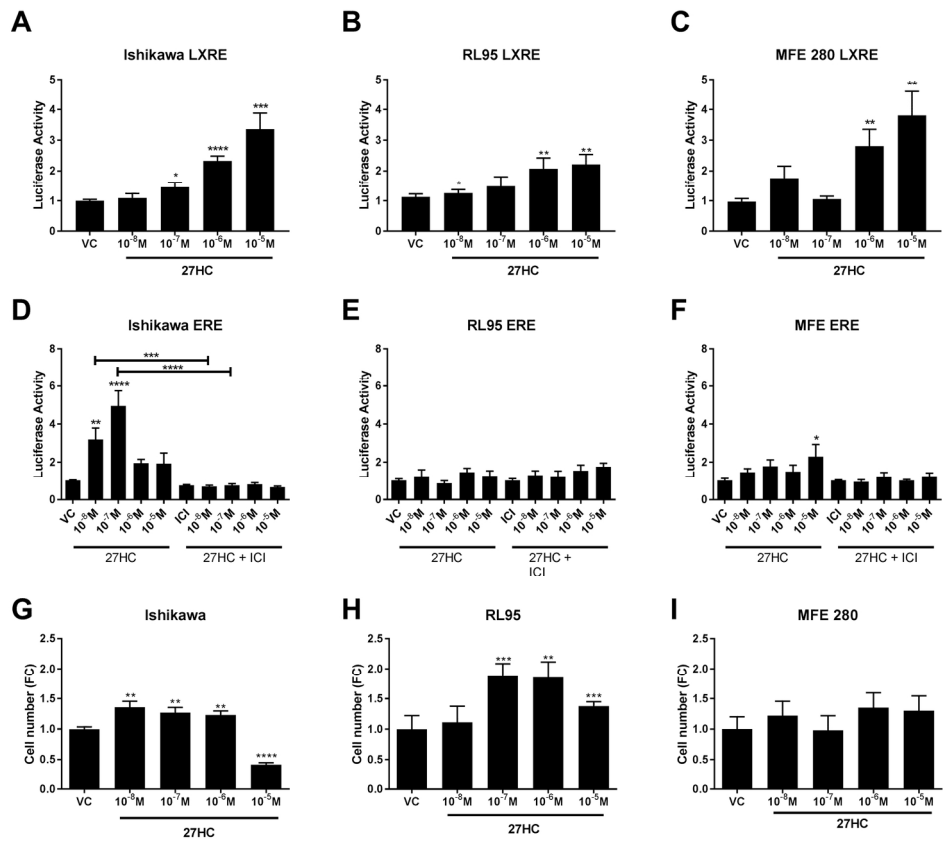


Figure 3. 27HC activates LXRE- and ERE-dependent transcription in Endometrial epithelial cancer cells and alters proliferation. The cholesterol metabolite 27-hydroxycholesterol (27HC) is the endogenous agonist for LXR and is also classified as selective estrogen receptor modulator. The impact of 27HC on LXRE- (A-C) and ERE-dependent (D-F) transcription was investigated by luciferase reporter assay in endometrial cancer cell lines; Ishikawa, RL95 and MFE280. 27HC significantly increased LXRE-dependent transcription in a dose-dependent manner in each endometrial cancer cell line. 27HC stimulated ERE-dependent transcription only at lower concentrations and was significantly increased by  $10^{-8}$  M 27HC ( $p < 0.01$ ) and maximally stimulated by  $10^{-7}$  M 27HC ( $p < 0.0001$ ). The 27HC effect was abrogated by co-incubation with the antiestrogen Fulvestrant (ICI 182,780; ICI) at all concentrations of 27HC (D). 27HC did not increase ERE-dependent transcription in RL95 (E) and was only increased by  $10^{-5}$  M 27HC ( $p < 0.05$ ) in MFE280 cells (F). Cell proliferation was assessed by CyQuant® direct proliferation assay in each cell line (G-I). Proliferation of Ishikawa cells was increased by  $10^{-8}$  M ( $p < 0.01$ ),  $10^{-7}$  M ( $p < 0.01$ ) and  $10^{-6}$  M ( $p < 0.01$ ) 27HC but decreased by  $10^{-5}$  M 27HC ( $p < 0.0001$ ; G). Proliferation of RL95 cells was increased by  $10^{-7}$  M ( $p < 0.001$ ),  $10^{-6}$  M ( $p < 0.01$ ) and  $10^{-5}$  M ( $p < 0.001$ ) 27HC (H). 27HC did not affect proliferation in MFE280 cells (I). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . One sample t test and a theoretical mean of 1. All data are presented as mean  $\pm$  SEM.

174x153mm (300 x 300 DPI)

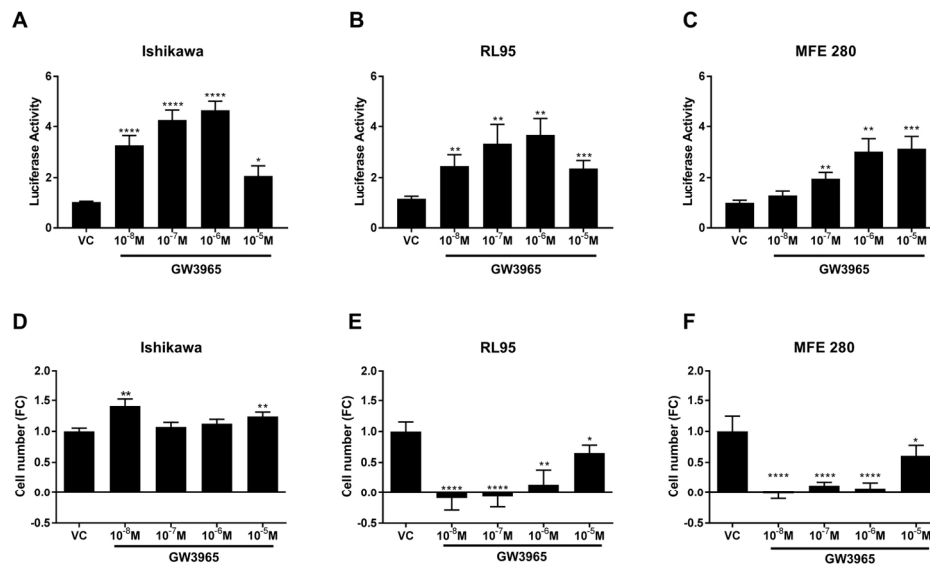


Figure 4. LXR agonist GW3965 activates LXRE-dependent transcription and alters proliferation in Endometrial epithelial cancer cells. The impact of the LXR synthetic agonist GW3965 on LXRE-dependent transcription (A-C) and on cell proliferation (D-F) was assessed in endometrial cancer cell lines; Ishikawa, RL95 and MFE280. GW3965 significantly increased LXRE-dependent transcription in a dose-dependent manner in each endometrial cancer cell line. GW3965 significantly increased LXRE-dependent transcription at all concentrations assessed and was maximally increased by 10-6M GW3965 in Ishikawa cells ( $p < 0.0001$ ) (A) and RL95 cells ( $p < 0.01$ ) (B). LXRE-dependent transcription and was not increased by 10-8M GW3965 but maximally increased by 10-5M 27HC ( $p < 0.001$ ) in MFE280 cells (C). Cell proliferation was assessed by CyQuant® direct proliferation assay in each cell line (D-F). Proliferation of Ishikawa cells was increased by 10-8M ( $p < 0.01$ ) and by 10-5M 27HC ( $p < 0.01$ )(D). In contrast, proliferation of RL95 cells was decreased by 10-8M ( $p < 0.001$ ), 10-7M ( $p < 0.001$ ), 10-6M ( $p < 0.01$ ) and 10-5M ( $p < 0.05$ ) GW3965 (E). Proliferation of MFE280 cells was decreased by 10-8M ( $p < 0.001$ ), 10-7M ( $p < 0.0001$ ), 10-6M ( $p < 0.0001$ ) and 10-5M ( $p < 0.05$ ) GW3965 (I). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . One sample t test and a theoretical mean of 1. All data are presented as mean  $\pm$  SEM.

156x93mm (300 x 300 DPI)